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Bylund, James Eugene, Ph.D.

The University of Nebraska - Lincoln, 1988

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ENZYME STUDIES AND SALT SENSITIVE MUTANT CHARACTERIZATION IN RELATION TO THE HALOTOLERANCE OF *HALOMONAS ELONGATA*

by

James E. Bylund

A DISSERTATION

Presented to the Faculty of

The Graduate College in the University of Nebrasksa

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Biological Sciences

Under the Supervision of Professor Eugene L. Martin

Lincoln, Nebraska

December, 1988

TITLE

Enzyme Studies and Salt Sensitive Mutant Characterization in Relation to the Halotolerance of Halomonas elongata.

BY

James E. Bylund

APPROVED	DATE
Dr. Eugene L. Martin	9-19-88
Dr. Thomas L. Thompson	9-19-88
Dr. John K. Dyer	9-19-88
Dr. John Osterman	9-19-88
Dr. Lloyd B. Bullerman	9-19-88

SUPERVISORY COMMITTEE

GRADUATE COLLEGE **UNIVERSITY OF NEBRASKA**

ENZYME STUDIES AND SALT SENSITIVE MUTANT CHARACTERIZATION IN RELATION TO THE HALOTOLERANCE OF *HALOMONAS ELONGATA*

James E. Bylund, Ph.D.

University of Nebrasksa-Lincoln, 1988

Advisor: Eugene L. Martin

The cytoplasmic enzyme alanine dehydrogenase (EC 1.4.1.1) and the extracellular enzymes alkaline and acid phosphatases (EC 3.1.3.1 and EC 3.1.3.2, respectively) from *Halomonas elongata* were partially purified from cells grown in an L-alanine defined medium with NaCl at 0.05, 1.37 and 3.4 M.

The activity profile for alanine dehydrogenase isolated from the three cell samples showed optimal activity at a NaCl concentration corresponding to the internal sodium concentration for each sample. Enzyme isolated from 0.05 M NaCl cells had highest activity at 0-20 mM NaCl; while enzyme isolated from 1.37 and 3.4 M NaCl cells had optimal activity at 340 and 500-600 mM NaCl respectively. Enzyme activity decreased when KCl or LiCl was substituted for NaCl. Polyacrylamide gel electrophoresis followed by histochemical staining revealed that 0.05 and 1.37 M NaCl cell extracts had two bands of activity (isozymes), while the 3.4 M NaCl cell extracts exhibited a single activity band.

Alkaline (AlP) and acid (AcP) phosphatases were cytochemically localized on the cell envelope. Enzyme assays were conducted at pH 9.0 (AlP) and 5.0 (AcP) with varying

concentrations of NaCl, KCl or LiCl in the assay buffer. Results show higher acid phosphatase activity compared to that of alkaline phosphatase and all enzyme activities were optimal at NaCl concentrations similar to the medium NaCl concentrations in which the cells were grown. However, all samples demonstrated strong activities at 4.0 M NaCl and at some KCl concentrations. Enzyme activities decreased significantly (with exceptions) when KCl or LiCl was substituted for NaCl. Polyacrylamide gel electrophporesis follwed by histochemical staining for the phosphatases showed only one band for both enzymes from each cell sample grown at different NaCl concentrations.

Salt sensitive mutants of H. elongata were generated by frame shift mutagens and isolated by selecting for the inability to grow at high NaCl concentrations. Salt tolerance studies revealed that each mutant tested had the same salt tolerance profile. The salt tolerance of one mutant (Hm1) could be extended by the presence of large amounts of tryptophan and glycerol. Tryptophan pathway studies demonstrated that Hm1 had a salt sensitve conditional mutation in the trp operon, possibly in the trp B gene region. Transport experiments using [^{14}C]tryptophan and [^{14}C]aminoisobutyric acid showed that Hm1 had a diminished uptake of these compounds compared to the parent.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

INTRODUCTION

A physiological explanation of halotolerance of bacteria has been the subject of research for many years. Available studies lead one to believe that the mechanism of halotolerance is not due to one factor but rather to the sum total of the properties of various cell components. If these factors are numerous and complex in nature, then a true understanding of halotolerance may be difficult to achieve. However, if the underlying mechanisms of halotoerance are relatively few, then they may be easier to understand and to control.

Bacteria of the genus Halomonas were originally isolated from a solar salt facility on the island of Bonaire in the Netherlands Antilles. H. elongata was found throughout the facility, demonstrating an extensive tolerance to salt. Since this organism has the ability to grow over an extended salt range and has a nutritional requirement for sodium, Vreeland and Martin (1980) designated H. elongata a halotolerant bacterium. Investigations into the halotolerance of Halomonas include the internal solute composition (Vreeland et al., 1983), aminoisobutyric acid transport (Martin et al., 1983), and cell wall and phospholipid composition (Vreeland et al., 1984).

The deleterious effects of monovalent salts on biological macromolecules are well known. A study of the

effects of salts on enzymes of Halomonag would provide further insight into the mechanism of halotolerance of this organism. A conventional and potentially profitable approach to the question of salt tolerance would be the study of mutants having diminished salt tolerance. Characterization of such mutants could answer key questions concerning the halotolerant mechanisms of Halomonas.

This dissertation: (1) examines and compares the salt response profiles of the intracellular enzyme alanine dehydrogenase and the extracellular enzymes alkaline and acid phosphatase of H. elongata, and (2) explores the physiological characteristics of a salt sensitive mutant of H. elongata.

LITERATURE REVIEW

In microbiology sodium chloride at significant concentrations is usually thought of as an unhealthy, stressful environment for microorganisms. However microbiologists have found such salty environments as ancient salterns, the Great Salt Lake, the (not quite) Dead Sea, and other hypersaline systems the domain of halophilic and halotolerant microorganisms. These organisms, adapted to tolerate NaCl or to require it as a nutrient (Larsen, 1962), represent a diverse group of yeasts, molds, algae, cyanophytes, and bacteria. The tolerance range or requirement that an organism has for salt has been the basis for many classification schemes.

The first workable scheme (Larsen, 1962) categorized microorganisms into four groups based upon their increasing requirements for NaCl: nonhalophiles, slight (marine), moderate, and extreme halophiles. Obligate halophiles require salt to survive and facultative halophiles do not require salt, but are stimulated by its presence.

Kushner (1978, 1985) attempted to associate molar values with the adjectives slight, moderate, and extreme (Table 1). The problems arising from using this classification scheme is that many organisms overlap the boundaries between classifications depending on the medium composition and the temperature at which they are grown (Kushner, 1978). Researchers have also induced algae that

TABLE 1.

Salt Response of Different Microorganisms[a]

Category	Reaction	Examples
Nonhalophile	Grows best in less than 0.2 M salt	Most eubacteria and freshwater organisms
Slight halophile	Grows best in 0.2-0.5 M salt	Marine organisms
Moderate halophile	Grows best in 0.5-2.5 M salt. Organisms able to grow in < 0.1 M salt are facultative halophiles	Vibrio costicola Paracoccus halodenitrificans
Borderline extreme halophile	Grows best in 1.5-4.0 M salt	Ectothiorhodospira halophila Halobacterium volcanii
Extreme halophile	Grows best in 2.5-5.2 M salt	Halobacterium salinarium Halococcus morrhuae
Halotolerant	Nonhalophile that can tolerate salt If growth range extends above 2.5 M salt, it is extremely halotolerant	Halomonas elongata Solute-tolerant yeasts, molds and algae

[a] Adapted from Table 3 in Kushner (1978).

would not grow at high salt concentrations to thrive there; and others, that once needed higher salt, to grow at more moderate levels (Borowitzka et al.,1977), thus demonstrating that some organisms can change their response to salt.

Vreeland (1987) presents a different view of halotolerance and halophilism in microorganisms. This classification scheme is independent of the envionmental salt concentration at which the organism resides. This classification scheme is, instead, based on the differences between upper and lower limits of growth. Though this would seem to be an answer to the problems stated earlier there is still the question of halophilism vs. halotolerance.

Reed (1986) defines halophiles as organisms that have an unusual requirement for salt which separates them from other organisms; any organism that has a minimum requirement for salt greater than 0.5 M can be regarded as a halophile. Halotolerant organisms are defined as having the capability of sustained growth at a level of 1.0 M NaCl or greater, but no absolute requirement for salt in amounts greater than 0.5 M. Reed (1986) also states that further subdivision (e.g. moderately halotolerant and extremely halotolerant categories) is difficult because of the complexities of microbial responses to salt and osmotic pressures and other environmental parameters (e.g. temperature, pH, etc.) upon salt tolerances and salt requirements, as measured in the laboratory.

However they are classified, all microorganisms growing

in saline environments are faced with the problems of hyperosmotic conditions and the toxic effects caused by NaCl. Adaptations of halophilic and halotolerant organisms to their environments can be divided into three broad categories defined by Reed (1986) as being:

(1) Insulation. Adaptations that reduce the impact of the external environment upon the cell interior by preventing or limiting the entry of Na^+ and the exit of water are in this category.

(2) Protection. In this category are mechanisms that protect intracellular function and metabolic activity from the inhibitory effects of Na^+.

(3) Modification. This category includes those aspects of cellular metabolism which show optimal function in above normal salt conditions. Such processes show modification when compared to similar processes in salt sensitive cells.

These categories are not mutually exclusive. Halophilic and halotolerant organisms commonly employ a combination of adaptations from the categories listed above. Details for each category are given below.

Osmoregulation and cell membrane adaptations are categorized as insulation and should be considered a first line of defense. Growth in high salt environments is not so much a problem of solutes as it is of water availability (Brown, 1976; Kushner, 1978). Organisms living in such

environments are able to obtain and hold onto water via osmoregulation. It is recognized that halotolerance in microorganisms is directly related to the cell's ability to concentrate, to varying degrees, K^+, Na^+, and low molecular weight compounds intracellularly (Vreeland, 1987). Christian and Waltho (1961, 1962) showed that when Halococcus morruhae and Halobacterium cutirubrum were grown in 4.25 M NaCl, their cytoplasm contained 4.25 M KCl. The halophilic cyanobacterium Aphanothece halophytica as well as its halotolerant cousins also accumulate potassium ions as their primary mechanism of osmoregulation (Yopp et al., 1978; Brock, 1976; Batterton and Van Baalen, 1971). Yopp et al. (1978) also discovered that the glycerol concentration in these organisms increased with increasing medium salt concentration. Eukaryotic algae and yeast have been found to concentrate polyols such as glycerol, mannitol, and arabinitol as well as potassium ions as osmoregulators (Fig. 1) The marine algae Dunaliella tertiolecta and the halophilic algae D. viridis increase their glycerol concentration proportional to the extracellular salt increase (Borowitzka and Brown, 1974). Asteromonas gracilis is a halotolerant algae with a growth range that extends to 4.5 M NaCl for which it increases its glycerol content accordingly (Ben-Amotz and Grunwald, 1981). Reed (1980) has shown that the internal mannitol concentration of a halotolerant marine alga varies as a function of external salinity. The halotolerant and osmotolerant yeastsDebaromyces hansenii and

Fig. 1. Representative examples of organic osmoregulators: (a) mannitol; (b) glycerol; (c) glutamate; (d) glycine betaine; (e) proline.